A Natural Diels-Alder Biocatalyst Enables Efficient [4 + 2] Cycloaddition Under Harsh Reaction Conditions

Carl O. Marsh,[a,b] Nicholas R. Lees,[c] Li-Chen Han,[b,c] Matthew J. Byrne,[a,b] Sbusisiwe Z. Mbatha,[c] Laurence Maschio,[a,b] Sebastian Pagden-Ratcliffe,[a] Phillip W. Duke,[d] James E. M. Stach,[e] Paul Curnow,[a,b] Christine L. Willis,[b,c] and Paul R. Race*[a,b]

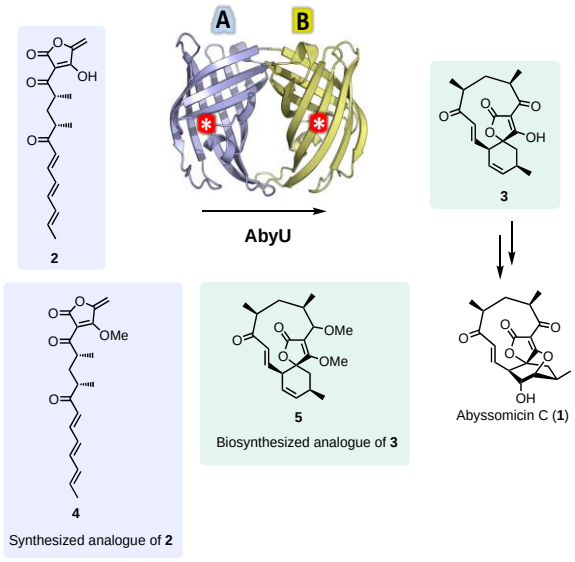
Abstract: Carbon-carbon bond formation is a fundamental transformation in both synthetic chemistry and biosynthesis. Enzymes catalyze such reactions with exquisite selectivity which often cannot be achieved using non-biological methods but may suffer from an intolerance of high temperature and the presence of organic solvents limiting their applications. Here we report the thermodynamic and kinetic stability of the *β*-barrel natural Diels-Alderase AbyU, which catalyzes formation of the spirotetronate core of the antimicrobial natural product abyssomicin C, with creation of 3 new asymmetric centers. This enzyme is shown to catalyze [4 + 2] cycloadditions at elevated temperature (up to 65 ºC), and in the presence of organic solvents (MeOH, CH_3CN and DMSO) and the chemical denaturant guanidinium hydrochloride, revealing that AbyU has potential widespread value as a biocatalyst.

Enzymes are now considered indispensable components of the synthetic chemistry toolkit and have become the method of choice for the preparation of a multitude of chiral molecules of pharmaceutical, agrochemical and industrial importance.[1] In particular, biocatalysts that perform carbon-carbon (C-C) bond forming reactions are in high demand, as they enable the rapid, enantioselective assembly of complex organic scaffolds.[2] One of the major obstacles to the use of enzymes in organic synthesis is their thermodynamic and structural instability under denaturing reaction conditions, properties that have restricted their use to chemo-enzymatic processes performed at ambient temperatures and in aqueous solvent.[3,4] The use of stabilizing mutations or post-translational modifications to circumvent this problem has yielded some successes.[5,6] However, these approaches are far from routine, and there remains a multitude of C-C bond forming reactions for which access to highly-stable biocatalysts would have significant impact.

One such example is the Diels-Alder reaction, a [4 + 2] cycloaddition that forms two C-C bonds and up to 4 stereocenters in a single step.[7] The development of Diels-Alderases as biocatalysts is an important goal, enabling access to a wide variety of high-value, structurally complex bioactive molecules. Although progress has been made in the identification and characterization of natural and *de novo* designed 'Diels-Alderases', these enzymes generally exhibit low catalytic efficiencies, restrictive substrate selectivities, and poor thermodynamic and kinetic stabilities, limiting their usefulness as tools in synthesis.[8,9]

In a previous study we reported the identification and characterization of the natural Diels-Alderase AbyU from the abyssomicin C (**1**) biosynthetic pathway, which catalyzes formation of the spirotetronate core of the antimicrobial natural product abyssomicin C, with creation of 3 new stereocenters. (Figure 1).[10,11] This *β*-barrel enzyme was shown capable of catalyzing [4 + 2] cycloadditions *in vitro*, with a > 4 x 10^4 fold enhancement in the rate of conversion of the linear diketone **4**, a synthetic analogue of the natural substrate **2**, to spirocyclic **5,** an analogue of the authentic product **3** (Figure 1).

Figure 1. AbyU catalyzed [4 + 2] cycloaddition reaction, synthetic substrate analogue (**4**), biosynthesized cycloaddition product (**5**), and crystal structure of



the enzyme (PDB 5DYQ). Individual *β*-barrel monomers that comprise the AbyU dimer are colored blue and yellow, with enzyme active sites indicated by white asterisks in red circles.

[a] C. O. Marsh, Dr M. J. Byrne, L. Maschio, S. Pagden-Ratcliffe, Dr P. Curnow, Dr P. R. Race
School of Biochemistry, University of Bristol,
Bristol, BS8 1TD (UK)
E-mail: Paul.Race@bristol.ac.uk
[b] C. O. Marsh, Dr L-C. Han, Dr M. J. Byrne, L. Maschio, Dr P. Curnow, Prof. C. L. Willis, Dr P. R. Race
BrisSynBio Synthetic Biology Research Centre, University of Bristol,
Bristol, BS8 1TQ (UK)
[c] Dr N. R. Lees, Dr L-C. Han, S. Z. Mbatha, Prof. C. L. Willis
School of Chemistry, University of Bristol,
Bristol, BS8 1TS (UK)
[d] P. W. Duke
Defense Science and Technology Laboratory,
Porton Down, SP4 0JQ (UK)
[e] Dr J. E. M. Stach
School of Natural and Environmental Sciences, Newcastle University, Newcastle-upon-Tyne, NE1 7RU (UK),
and
Centre for Synthetic Biology and the Bioeconomy, Newcastle University, Newcastle-upon-Tyne, NE2 4AX (UK)

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Further, the ability of AbyU to accept and act upon non-natural substrates was demonstrated. Although these data hint at the potential value of AbyU as a general Diels-Alder biocatalyst, they reveal little about the ability of the enzyme to catalyze [4 + 2] cycloaddition under conditions routinely employed during organic synthesis, such as elevated temperature and with organic solvent.

Motivated to explore the broader utility of AbyU in organic synthesis, we sought to assess the stability and catalytic activity of this enzyme under conditions of high temperature and in the presence of chemical denaturants and organic solvents. In the first instance a folding study was conducted to establish the breadth of conditions under which the enzyme retains structural integrity. Initially, we examined the thermal unfolding behavior of AbyU using circular dichroism (CD) spectroscopy. This enabled the quantitation of protein secondary structure composition as a function of increasing temperature. The thermal denaturation of AbyU was investigated by collecting far-UV CD spectra (180 - 280 nm) from a phosphate buffered solution of purified recombinant enzyme (Figure S1), at temperatures ranging from 25 - 95 °C, in 5 °C increments (Figure 2). The far-UV CD spectrum of AbyU exhibits a maxima at 195 nm and a minima and 217 nm (Figure 2A). These features are typical of proteins with high beta-strand content[12] and are consistent with the published crystal structure of the enzyme[11] (Figure 1, PDB 5DYQ). Thermal denaturation of AbyU gives a melting temperature of 61.5 ± 0.3 °C. The enzyme undergoes a rapid, irreversible unfolding above this threshold (Figure 2B). The thermal unfolding behavior of AbyU is consistent with a two-state process with no observable intermediates. Unfolding progresses *via* the formation of a quickly resolved, largely disordered polypeptide chain that is unable to spontaneously refold (Figure S2). The irreversibility of this process precludes calculation of the thermodynamic parameters of AbyU unfolding.

Next, we sought to investigate the effect of temperature on the AbyU catalyzed [4 + 2] cycloaddition reaction. *In vitro* enzyme assays were performed as previously described, monitoring the conversion of the substrate analogue **4**, to the spirocyclic product **5**, spectrophotometrically at 300 nm.[11] Reaction rates were measured at 10 °C intervals from 25 - 85 °C with non-AbyU catalyzed reaction rates subtracted (Figure 2C). Analysis of the AbyU reaction product by HPLC indicated that a single product had been formed with mass (*m/z* = 367) and ^{1}H-NMR consistent with **5** (Figures S3 and S4, and SI). Control experiments were carried out to monitor non-enzyme catalyzed [4 + 2] cycloaddition at elevated temperatures. AbyU retains catalytic activity at temperatures of up to 65 °C, highlighting its suitability for use in mid-range temperature processes. Between 45 - 65 °C there is a sequential decrease in enzyme activity, which correlates with a loss of secondary structure (Figure 2D). No catalytic activity was observed at temperatures > 65 °C, consistent with our CD data, which indicates a complete loss of secondary structure and the adoption of a predominantly unfolded state.

To complement our thermal denaturation studies, the unfolding behavior of AbyU in the presence of the chemical denaturant guanidinium hydrochloride (GuHCl) was investigated (Figure 3). AbyU unfolding was monitored using both CD spectroscopy (Figure 3A) and fluorescence spectroscopy

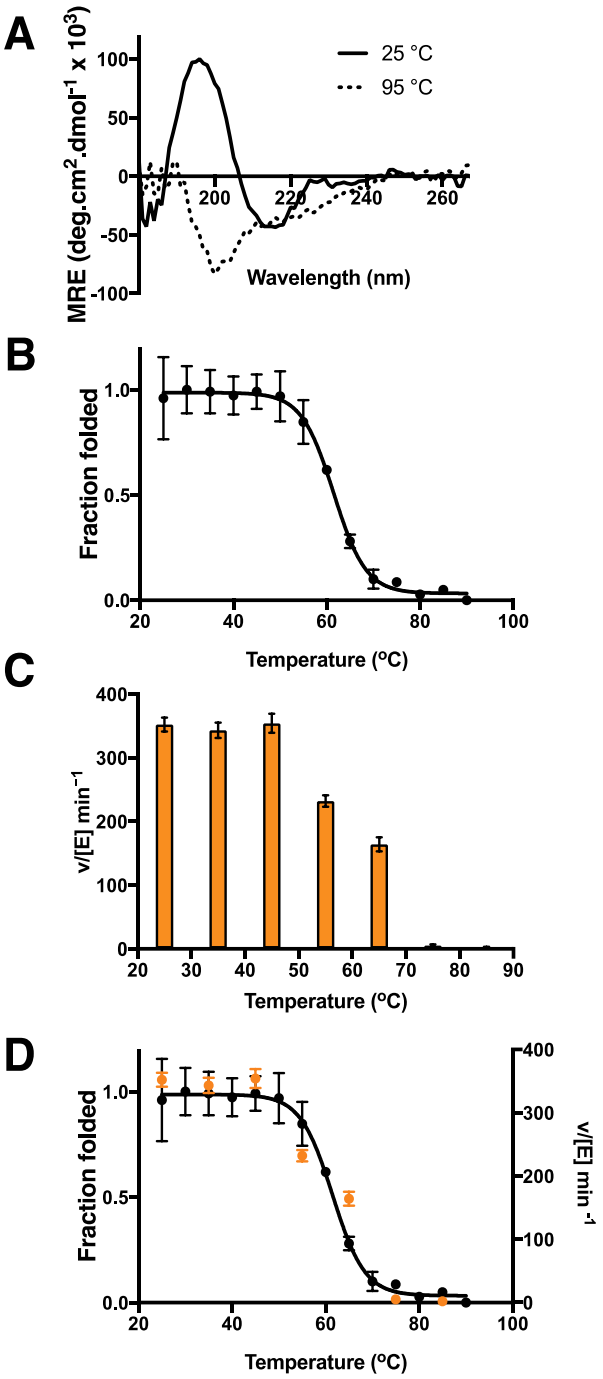

Figure 2. Thermal unfolding and kinetic studies of AbyU. (**A**) CD spectra of AbyU at 25 °C (solid line) and 95 °C (dotted line). (**B**) Thermal unfolding of AbyU as monitored by CD spectroscopy. Fraction folded values are calculated from mean sample ellipticity readings at 200 nm derived from three repeats of the same experiment. Errors bars are standard errors from the mean. Data are fitted to a Boltzmann sigmoid equation assuming a two-state model. (**C**) Reaction rates for the AbyU catalyzed cyclization of diketone **4** to give cycloaddition product **5** at increasing temperature. Rates are mean values calculated from 3 repeats of the same experiment. Error bars are standard errors from the mean. (**D**) Superposition of data in (**B**) and (**C**).

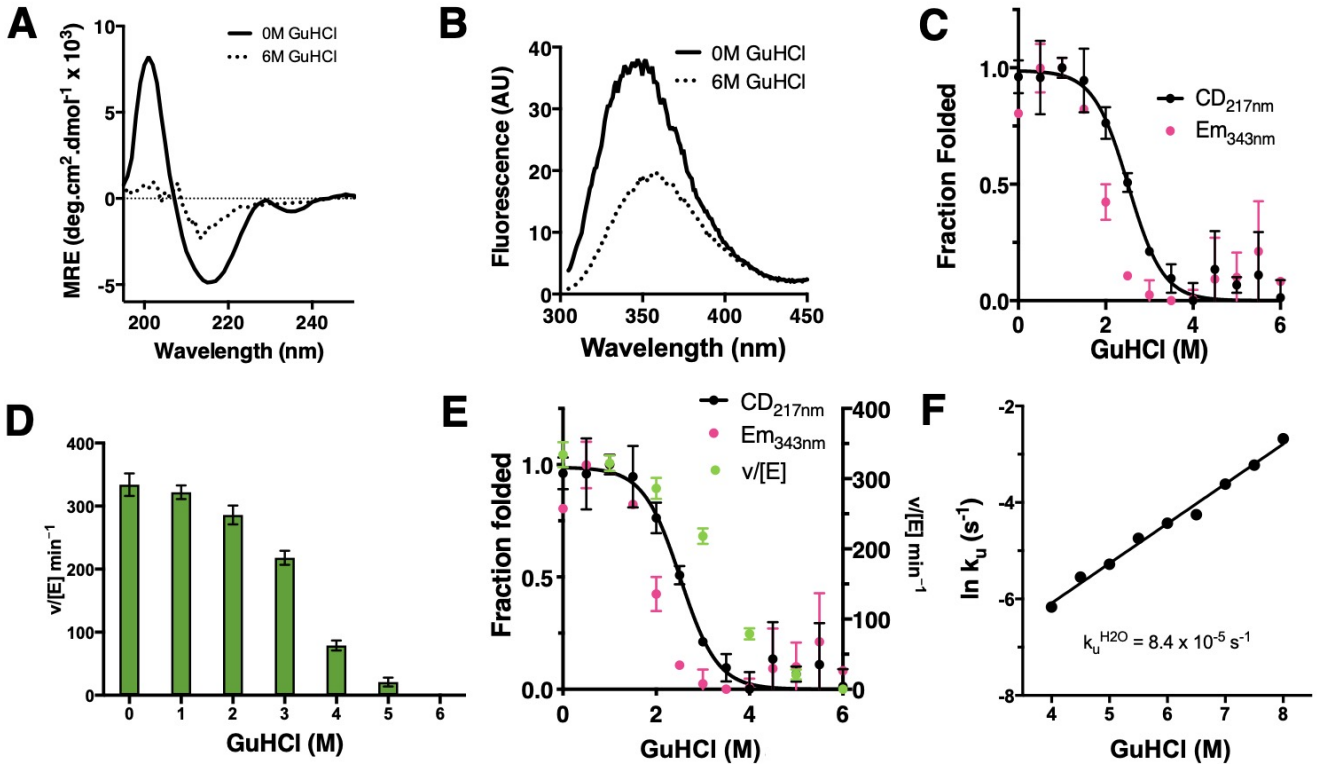

Figure 3. Chemical denaturation studies of AbyU. (**A**) CD spectra of AbyU in buffer containing 0 M (solid line) or 6 M (dotted line) GuHCl. (**B**) Fluorescence spectra of AbyU in buffer containing 0 M (solid line) or 6 M (dotted line) GuHCl. (**C**) GuHCl induced unfolding of AbyU as monitored using CD spectroscopy (black) and fluorescence spectroscopy (pink). Fraction folded values from the CD analysis are calculated from mean sample ellipticity readings at 200 nm derived from three repeats of the same experiment and are fitted to a the Fersht equation (SI). Fraction folded values from the fluorescence analysis are calculated from the emission signal intensity at 343 nm (SI). Errors bars are standard errors from the mean. (**D**) Reaction rates for the AbyU catalyzed cyclization of diketone **4** to give cycloaddition product **5** at increasing concentrations of GuHCl. Rates are mean values calculated from 3 repeats of the same experiment. Error bars are standard errors from the mean. (**E**) Superposition of data in (**C**) and (**D**). (**F**) Kinetic unfolding of AbyU at increasing GuHCl concentration.

(Figure 3B) in the presence of increasing GuHCl concentration (0 - 6 M). AbyU contains three tryptophan residues, two of which are located on the outer surface of the enzyme's barrel fold (W92 and W103), and a third which resides within the enzyme active site (W124). This latter residue provides a convenient probe for measuring the degree of solvation of the predominantly hydrophobic enzyme active site during unfolding. Consistent with our thermal denaturation studies, chemical denaturation of AbyU is a non-reversible two-state process, which proceeds without the formation of intermediate species (Figures 3C and S2). Deconvolution of GuHCl induced unfolding of AbyU at equilibrium, as monitored by fluorescence spectroscopy, gave $\Delta G = -3.7 \pm 0.8$ kcal.mol^{-1} and a cooperativity coefficient (*m*) of 1.5 ± 0.3 kcal.mol^{-1}.M^{-1}. The kinetic activity of AbyU in the presence of increasing GuHCl concentration correlates well with the proportion of folded enzyme in each sample (Figures 3D and 3E). There is no evidence of catalytic activity in 6 M GuHCl, consistent with a complete loss of protein structure. Kinetic characterization of the chemical unfolding process gave $k_U^{H2O} = 8.4 \times 10^{-5}$ s^{-1} (Figures 3F and S5).

To complete our study of AbyU thermodynamic and kinetic stability, we next assessed the tolerance of the enzyme to organic solvents. Changes in solvent condition greatly influence key aspects of protein stability and folding, and resulting thermodynamic and structural effects can be readily measured using spectroscopic methods. We selected three solvents commonly employed in organic synthesis; the polar aprotic solvents acetonitrile (MeCN) and dimethyl sulfoxide (DMSO), and the polar protic solvent methanol (MeOH), and performed fluorescence based equilibrium unfolding titrations and *in vitro* activity assays using recombinant AbyU (Figure 4).

AbyU shows considerable tolerance to the organic solvents tested as indicated by retention of catalytic activity and structural integrity. Remarkably, the enzyme is able to catalyze [4 + 2] cycloadditions in solvent concentrations of up to 80% v/v MeCN (Figure 4A), DMSO (Figure 4B) and MeOH (Figure 4C). There is good correlation between AbyU reaction rate and corresponding fluorescence signals, indicating that kinetic competency is intimately linked to adoption of a fully folded state. AbyU retains > 50% foldedness in MeCN concentrations of up to 70% v/v, and > 70% foldedness in 80% DMSO and MeOH v/v (Figure 4D). These data give strong support for the feasibly of using AbyU under conditions of high solvent concentration, which together with the thermal and chemical denaturant tolerances reported in this study demonstrates the compatibility of AbyU with harsh reaction conditions.

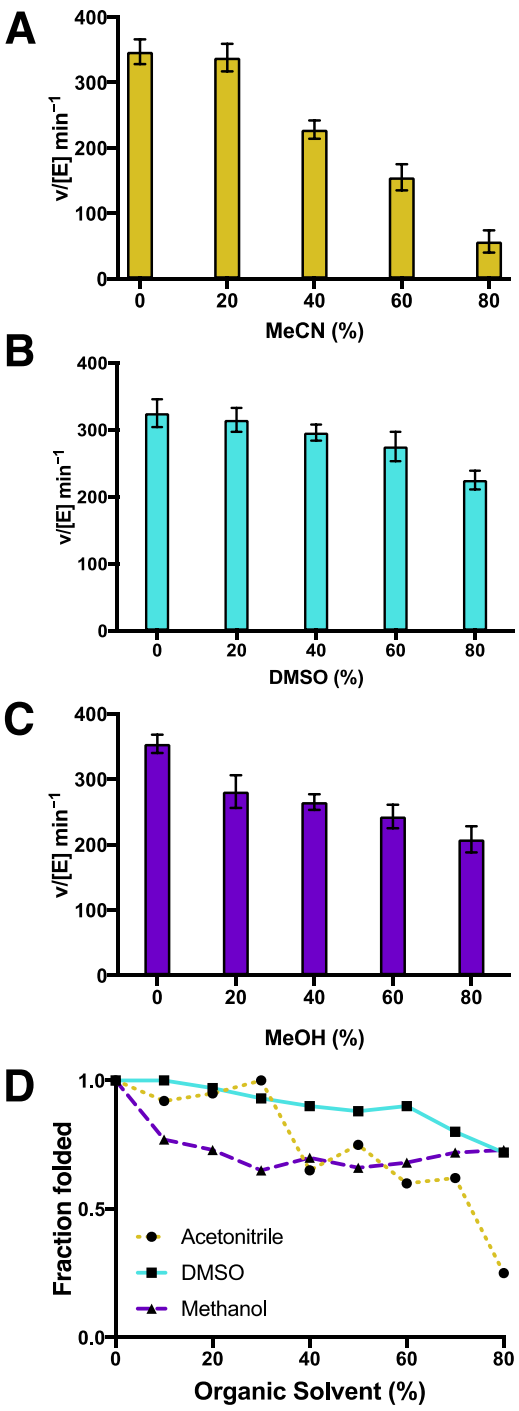

Figure 4. Effect of organic solvents on AbyU. Reaction rates for the AbyU catalyzed cyclization of diketone **4** to give cycloaddition product **5** at increasing concentrations of (**A**) MeCN, (**B**) DMSO and (**C**) MeOH. Rates are mean values calculated from 3 repeats of the same experiment. Error bars are standard errors from the mean. (**D**) Unfolding profiles for AbyU in increasing concentrations of MeCN (yellow), DMSO (cyan) and MeOH (purple).

In summary, we report herein the first detailed study of the stability and folding behavior of a Diels-Alder biocatalyst, the *β*-barrel enzyme AbyU. This polypeptide is shown to retain overall structure and catalytic function under a range of reaction conditions, including those typically employed in organic synthesis. The robustness of AbyU enables [4 + 2] cycloadditions to be performed with high catalytic efficiency at temperatures up to 65 °C, and in the presence of chemical denaturants and organic solvents. These findings further highlight the value of AbyU and related *β*-barrel enzymes as industrially relevant biocatalysts, providing a benchmark for the development of engineered variants of these proteins with enhanced thermal and chemical stabilities.

Experimental Section

A detailed description of all experimental procedures including gene cloning, protein expression and purification, folding studies, substrate synthesis, and enzyme assays are provided in SI Materials and Methods.

Acknowledgements

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Keywords: Biocatalysis • Diels-Alderase • natural products • protein folding • cycloaddition

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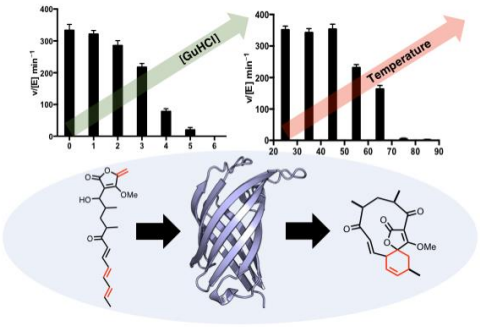
Feel the burn: Exploration of the thermodynamic and kinetic stability of the natural Diels-Alderase AbyU establishes the operational limits of this biocatalyst, revealing the enzyme's capacity to catalyze [4 + 2] cycloadditions under harsh reaction conditions.



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